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Resistance Induction and Enhanced Tuber Production by Pre-inoculation with Bacterial Strains in Potato Plants against *Phytophthora infestans*

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Efficacy of resistance induction by the bacterial isolates *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412), which were isolated from the rhizosphere of plants growing in Jeju Mountain, were tested in a greenhouse. The disease severity caused by *Phytophthora infestans* was effectively reduced in the potato plants pre-inoculated with bacterial isolates compared with those of the untreated control plants growing in a greenhouse. In order to estimate the level of protection by the bacterial isolates, Mancozeb WP (Diesen M®, Kyong nong) and DL-3-amino butyric acid (BABA) were pre-treated, whereas Dimethomorph WP (Forum®, Kyong nong) and phosphonic acid (H_3PO_3) were post-treated the challenge inoculation with the pathogen. Disease severities of chemical pre-treated as well as post-treated plants were reduced compare to those of the untreated. The disease reduction in the plants pre-treated with Mancozeb WP was the highest, whereas that of post-treated with Dimethomorph WP was the lowest. The yields of plants pre-inoculated with three bacterial isolates were greatly increased than those of control plants. These results suggest that biological control by bacterial isolates might be an alternative strategy against late blight disease in potato plants growing in greenhouse.

KEYWORDS: **DL-3-amino butyric acid (BABA), Dimethomorph WP (Forum®), Induced systemic resistance, Mancozeb WP (Diesen M®), Phosphonic acid (H_3PO_3), *Phytophthora infestans*, Plant growth promoting rhizobacteria (PGPR), Potato**

Late blight disease caused by the oomycete fungus *Phytophthora infestans* has been often occurred in potato cultivating area under cool and rainy weather conditions and potato can be severely damaged by the late blight disease (Erwin and Ribeiro, 1996).

The pathogen survives in volunteer potato plant material in the fields. It becomes the primary source of inoculum in the next year. Under favorable conditions, potato plants are infected by the overwintering inoculum (Jones *et al*., 1991). In infected plant tissues, the fungus sporulates and forms sporangia on typical sporangiophores. The sporangia drift off by wind or are dispersed by rain and cause infections by releasing zoospores which can again rapidly infect wet leaves, stems and fruits under optimal temperature and humidity conditions (Erwin and Ribeiro, 1996). Beside asexual sporulation, the late blight fungus is able to perform sexual recombination resulting in oospore production. The sexual life cycle may affect the epidemiology and increase of selection of fungicide resistant strains in the population of the late blight fungus in potato growing regions (Fry and Goodwin, 1995).

Late blight is one of the diseases, which are difficult to control in the field. Generally, varieties which are resistant to only one or a few races of the late blight fungus, can become susceptible when they are attacked by new virulent races of the fungus. Beside the vertical resistance, some varieties possess horizontal resistance of varying degree, which is effective against all races of the blight fungus. Cultivating resistant varieties is not sufficient to control the late blight disease, since under favorable conditions *P. infestans* can severely infect even resistant varieties. If the appropriate fungicides such as mancozeb, metalaxyl, or combination of both or other fungicides are properly applied, the late blight can be kept under control. Using a simulation program it is possible to predict the first and subsequent infection periods by analyzing the data of temperature, relative humidity and rainfall, which are daily recorded in the cultivating area (Agrios, 2005). Therefore, late blight can be more or less successfully controlled by proper applications of appropriate fungicides according to the late blight forecast.

One of the strategies for plant protection against late blight disease may be using crops expressing a systemic induced resistance, which can be triggered in the plant by pre-inoculation with plant growth promoting rhizobacteria (PGPR) (van Loon *et al*., 1998a). The PGPR-mediated resistance has been defined as induced systemic resistance (ISR) (van Loon *et al*., 1998a). The treatment with PGPR may enhance the plant own defense mechanism, which result in expression of systemic resistance on the aerial part of the plants (van Loon *et al*., 1998a). Moreover, for expression of resistance the PGPR need not be contact with plant pathogens and the PGPR can grow well in the rhizosphere. Therefore, using PGPR is one of

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the possible strategies for plant protection in the field (van Loon *et al*., 1998a).

In the previous study bacterial strains were isolated from the rhizosphere of the plant growing in Jeju and the anti-fungal activities of the isolates against several plant pathogens were tested and selected (Lee *et al*., 2003). In this study aiming for the selection of an effective ISR inducing agent, efficacy of the selected bacterial isolates *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) against late blight caused by *P. infestans* was tested in potato plants. Furthermore, to estimate the control efficacy of the bacterial isolates disease severities of plants treated with two resistance activators and two commercial fungicides were compared after inoculation with the late blight fungus. Additionally, the total weighs of tubers per potato plants were compared among the bacterial isolates pre-inoculated, resistance activators treated, and commercial fungicides treated plants.

Materials and Methods

Plants. Seed tuber of potatoes (*Solanum tuberosum* L. cv. Deajima) were grown in a polystyrene box (W × L × D = 31 × 51 × 20 cm) filled with perlite and peatmoss mixture (1 : 2, v/v) in a greenhouse at 25°C during the day and at 20°C during the night. Plants were grown in a greenhouse.

Treatment of bacterial isolates in the plants for triggering of ISR. The bacterial isolates *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) showing antifungal effect were selected to test triggering of ISR in plants. The strains were grown in tryptic soy agar at 28°C for 24 h. The concentration of bacterial strains was adjusted to be 1.0×10^7 colony forming unit (cfu)/*ml* according to the methods described by Park and Kloepper (2000).

Thirty *ml* of the bacterial suspension was soil-drenched per potato plants 7 days before challenge inoculation with *P. infestans*. For negative control, H_2O was applied on the potato plants instead of the bacterial suspension. In order to estimate the level of protection by the bacterial isolates, Mancozeb WP (Diesen M®, Kyong nong; 2,000 ppm) and DL-3-amino butyric acid (BABA; 10 mM) were pre-treated 7 days before challenge inoculation, whereas Dimethomorph WP (Forum®, Kyong nong; 1,000 ppm) and phosphonic acid (H_3PO_3; 2,000 ppm) were post-treated 7 days after the challenge inoculation with pathogen.

Three experiments were replicated with time intervals and every experiment carried out with 3 replications containing 8 plants each.

Challenge inoculation with pathogen. *P. infestans* (Mont.) de Bary was grown on V8 agar medium for 7 days at 15°C to induce sporangium formation. For the initiation of zoospore release from sporangia, 10 *ml* H_2O were added to the agar plate grown with the fungal mycelium. A spatula was used to remove air between hyphae so that sporangia were submerged in water. Then the plates were immediately placed in a refrigerator at 4°C until zoospores were released. The suspension containing zoospores was filtered through three times folded cheesecloth and the concentration of the zoospores was adjusted to 1.5×10^4 zoospores/*ml* for the inoculation of potato plants.

The zoospore suspension of *P. infestans* was sprayed on the aerial potato leaves 7 days after the treatment with the suspension of bacterial suspension or chemicals. The potato plants inoculated with suspension of *P. infestans* were covered with black polyethylene vinyl for 24 h for and keeping the relative humid to 100%.

Determination of increase of yield by bacterial isolates. To determine the increase of yield by the pre-inoculation with the bacterial isolates the seed tuber of potatoes were sown in plastic pots (Ø 25 cm). Thirty ml of the suspension of bacterial TRL2-3, TRK2-2 and MRL412 isolates was soil-drenched per potato plants at four weeks before harvest. For control, chemicals BABA as well as Mancozeb WP were also treated at the same time as the bacterial inoculation. The total weight of potato tubers harvested per plant was measured using a balance. The experiment was carried out with 3 replications containing 3 plants each.

Evaluation of resistance. Disease severity caused by *P. infestans* was determined at 14 days after challenge inoculation. The disease severities were established using the following scale: 0, no lesion; 1, up to ~20% of the leaf area blighted; 2, 20~40%; 3, 40~70%; 4, 70~90%; 5, plant entirely blighted. The disease severities of every branch were determined and presented the mean of the total scale numbers. Percentage protection against the disease was calculated as according to Cohen (1994) described as protection (%) = 100(1 – x/y) in which x and y are disease severity values in treated and control plants after challenge inoculation, respectively.

The data of disease severity caused by *P. infestans* and of total tuber weight were statistically analyzed using Duncan's multiple range tests.

Results

The bacterial isolate *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) showing antifungal activity in vitro test (Lee *et al*., 2003) was selected in order to determine their

resistance efficiencies against disease caused by *P. infestans* in potato plants. The lesion caused by late blight was well developed in the leaves of untreated control plants after inoculation with *P. infestans* and at 14 days the disease severity was over 80% (Fig. 1 and 2). Disease severity of late blight was significantly suppressed by the pre-inoculation with all bacterial isolates tested (Fig. 2). Although in the first experiment the protection rate of the TRK2-2 pre-inoculated plants was lower than those of other bacterial isolate pre-inoculated plants, similar protec-

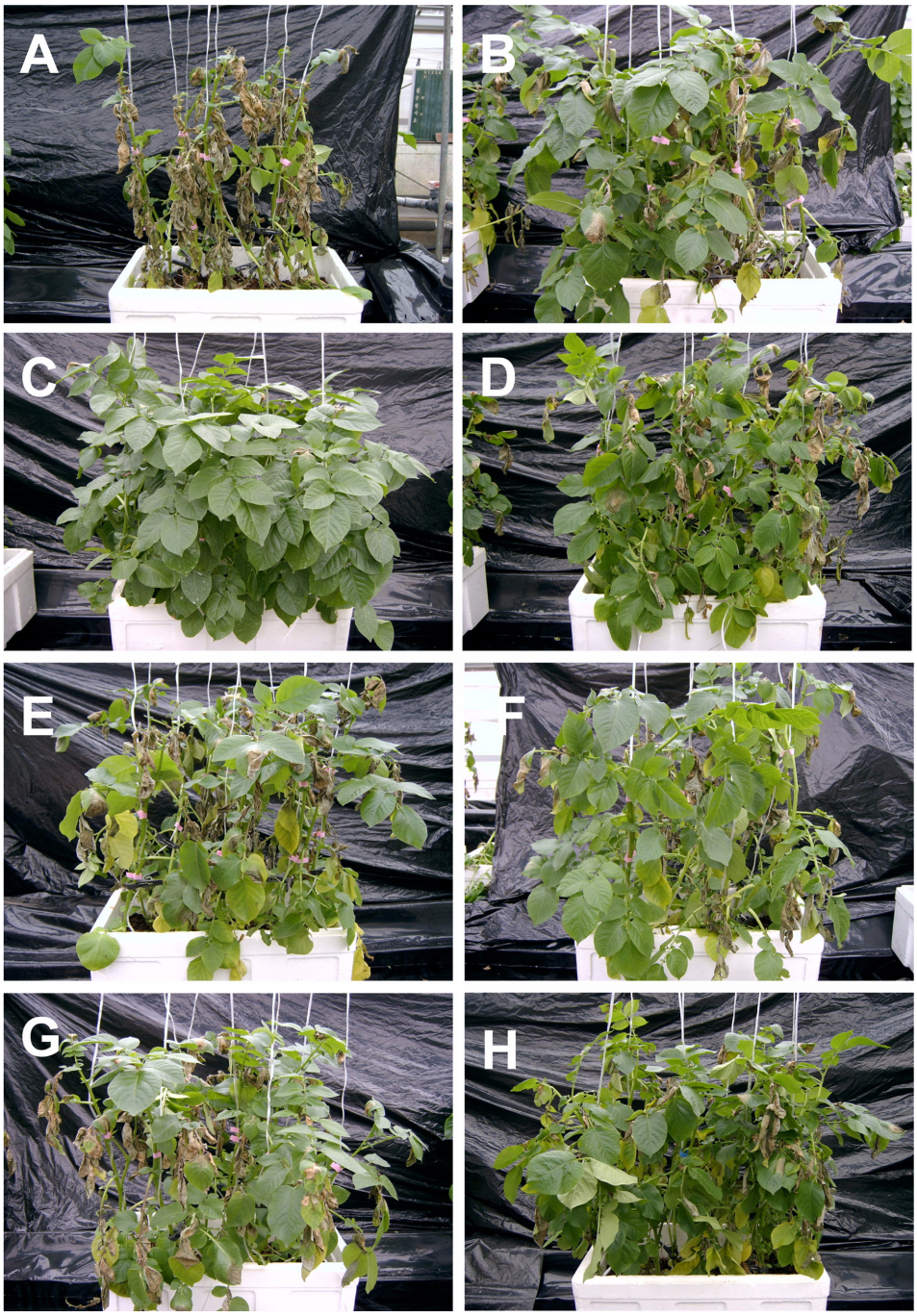

Fig. 1. Induction of systemically induced resistance in potato plants against late blight disease at 14 days after inoculation with *P. infestans* (1.0×10^4 zoospores/*ml*). The presented plants were (A) untreated control, (B) pre-treated 7 days before challenge inoculation with DL-3-amino butyric acid (BABA; 10 mM), (C) Mancozeb WP (Diesen M®, 2,000 ppm), (D) pre-inoculation 7 days before challenge inoculation with bacterial suspension of *Pseudomonas putida* (TRL2-3), (E) *Micrococcus luteus* (TRK2-2), (F) *Flexibacteraceae bacterium* (MRL412) with the concentration of 1.0×10^7 cfu/*ml* each, (G) post-treated 7 days after challenge inoculation with Dimethomorph WP (Forum® 1,000 ppm), and (H) phosphonic acid (H_3PO_3; 2,000 ppm).

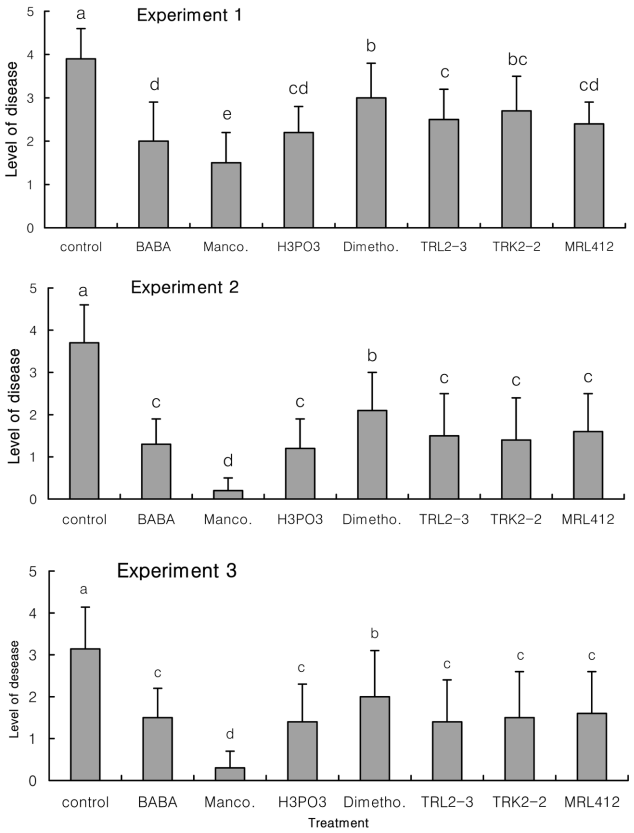

Fig. 2. Comparison of reduction of disease severities and Duncan's multiple range test in the leaves of potato plants by three selected bacterial isolates, by two resistance activators and by two commercial fungicides after challenge inoculation with *P. infestans* (1.0×10^4 zoospores/*ml*). Disease severity were estimated with the scale of infected leaves as following: 0, no lesion; 1, up to ~20% of the leaf area blighted; 2, 20~40%; 3, 40~70%; 4, 70~90%; 5, plant entirely blighted. The vertical bars indicate the standard deviation of the 3 replications each containing 8 plants per treatment.

tion rate of the disease severities were shown both in second and third experiments (Fig. 2).

The protection of late blight by pre-treated with chemical mancozeb was remarkable in all experiments (Fig. 2). The disease severity of pre-treated with BABA was significantly reduced compared to those of untreated plants (Fig. 2). The disease control efficacy by BABA was higher than those of bacterial isolates in the first experiment but similar in both second and third experiments (Fig. 2). Also, the post-treatment with phosphonic acid could reduce the disease severity as case of BABA pre-treated, although in the first experiment the protection rate was slightly lower (Fig. 2). Interestingly, protection rate by the post-treatment with Dimethomorph WP was the lower than those by three bacterial isolates, although disease severity was reduced compared to untreated control (Fig. 2).

Table 1. Comparison of total tuber weight of potato plants pre-inoculated/-treated with three bacterial isolates or with two chemicals and Duncan's multiple range test

Treament[a]	Total weight of tubers per plant (g)	Duncan's test
TRL2-3	269.3 ± 66.7[b]	a
TRK2-2	313.9 ± 52.3	a
MRL412	174.3 ± 42.8	b
Mancozeb	124.5 ± 20.1	bc
BABA	103.6 ± 16.5	bc
Control	76.4 ± 6.7	c

[a]The bacterial isolates TRL2-3, TRK2-2 and MRL412, which were *Pseudomonas putida*, *Micrococcus luteus* and *Flexibacteraceae bacterium* identified, respectively, and Mancozeb WP (Diesen M[®], Kyong nong) and DL-3-amino butyric acid (BABA) were pre-inoculated/-treated at four weeks before harvest.
[b]Mean of values ± standard deviation of the three replications.

The pre-inoculation with all three bacterial isolates caused an increase of yields of the potato tubers. Both bacterial isolates TRL2-3 and TRK2-2 could remarkably increase the total tubers per plants compared to those of control plants (Table 1). Also, the pre-inoculation with MRL412 could cause the significant increase of tuber production per plants (Table 1). Although yield increase of the plants pre-treated with both chemicals BABA and Mancozeb WP were not as high as the cases of TRL2-3 or TRK2-2, the total weight of tubers were slightly increased compared to that of untreated control (Table 1).

Discussion

Using microorganisms for disease control has been considered for many years because this strategy results in the reduction of chemical application. However, using the antagonistic microorganisms to control of plant diseases has not been always successful in the field, new strategy of the biological control such as using crop plants expressing an induced systemic resistance (ISR) has been looking for controling plant diseases (van Loon *et al*., 1998a). It may result in reduction of chemical application in the field. In this study to select an effective microorganism inducing ISR against plant diseases, the some rhizobacteria showing antifungal activity were tested with potato-late blight interaction systems.

In the potato plants growing in the green house culture ISR could be effectively triggered when three bacterial isolate were pre-inoculated (Fig 2). The bacterial concentration at 1.0×10^7 cfu/*ml* mediated most effectively resistance against late blight among the other ones (data not shown). In our previous study the control efficacies of TRL2-3 and TRK2-2 were also shown in cucumber plants after challenge inoculation with anthracnose pathogen *Colletotrichum orbiculare* (Jeun *et al*., 2004a). Similarly some PGPR strains such as *Serratia marcescens* or

Pseudomonas fluorescens could effectively induce systemic resistance in cucumber plants against anthracnose disease at certain concentration (Liu *et al.*, 1995).

The mechanisms of ISR have been compared with those of systemic acquired resistance (SAR) (Jeun *et al.*, 2004b), which has been studied in details (Sticher *et al.*, 1997). In contrast to SAR, some PGPR strains mediating systemic resistance have direct antifungal activity. In our previous study both bacterial isolates TRL2-3 and TRK2-2 showed direct antifungal effect in vitro test (Lee *et al.*, 2003). Another mechanism of expression of ISR is competition mineral element such as ion (Fe), which is easily captured by siderophores produced in PGPR (Maurhofer *et al.*, 1994; Van Loon *et al.*, 1997, 1998b). The resistance expression by competition of nutrient has not been reported in the plants expressing SAR, too. The other resistance mechanisms of ISR, however, seem to be similar with those of SAR, which is involved in the resistant gene *npr1* (Pieterse and van Loon, 1999).

DL-3 amino butyric acid (BABA) is well known as an activator in many plants (Cohen, 2002; Jeun and Park, 2003; Zimmerli *et al.*, 2000). In this study the pre-treatment with BABA could be caused the effective induction of systemic resistance (Fig. 2). Similarly, the disease severity was reduced in the plants post-treated with phosphonic acid (H_3PO_3) (Fig. 2). Disease control with phosphonic acid, especially against the disease caused by Oomycetes, has been already reported in some studies (Grant *et al.*, 1990; Guest *et al.*, 1995). In these all three experiments the disease control efficacy of the bacterial isolates were comparable to those of both ISR inducers (Fig. 2).

The pre-treatment of commercial fungicide Mancozeb WP was the best control strategy for protection of late blight in potato plants. However, the post-treatment of another fungicide Dimethomorph WP could not decrease the disease severity as the case of pre-inoculated with three bacterial isolates (Fig. 2). Based on these results late blight in potato plants may be effectively controlled by spraying a protective fungicide before break out of the disease. Furthermore, the disease control efficacy by the bacterial isolates was higher than those of fungicide Dimethomorph WP (Fig. 2). This result indicated the possibility of alternative disease control strategy using bacterial isolates triggering ISR in the potato in the green house.

A total weight of tubers was greatly increased in plants pre-inoculated with bacterial isolates TRL2-3 or TRK2-2 compared to those of untreated control plants (Table 1). Although the fungicide Mancozeb WP and resistance activator BABA caused increase of yield, the total weight of tubers of both chemical treated plants was not significantly different to that of untreated control plants (Table 1). These results suggest that both the protection efficacy against late blight and yield increase of tubers might be expressed in potato plants cultivating with the green house. The enhancement of plant growth and resistance indcution mediated by rhizobacteria have been reported in many host-pathogen interactions (Gamo and Ahn, 1991; Kloepper *et al.*, 1980).

In summary, the bacterial isolate TRL2-3, TRK2-2 and MRL412 could trigger ISR in potato plants against late blight. Although the ISR by the isolate was not higher compared to those of commercial fungicide Mancozeb, it is suggested that the protection by using microorganism may be useful in a green house, where chemical application is forbid. For this purpose, more research concerning ISR should be carried out.

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